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(54) Title: FLUORENYL-HYDROXAMIC DERIVATIVES ENDOWED WITH IMMUNOSUPPRESSIVE AND ANTI-INFLAMMATORY ACTIVITY

(57) Abstract

The present invention refers to hydroxamic derivatives of fluorenylaminoacids and their use as immunosuppressant and anti-inflammatory agents.

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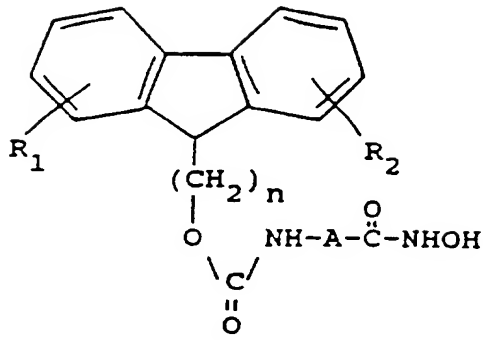
FLUORENYL-HYDROXAMIC DERIVATIVES ENDOWED WITH IMMUNO-SUPPRESSIVE AND ANTINFLAMMATORY ACTIVITY

The present invention refers to hydroxamic derivatives of fluorenylaminoacids and their use as immunosuppressant and antinflammatory agents.

Since long the fluorenylaminoacids have been known as intermediates in the peptide synthesis, and recently they were found also to be endowed with antinflammatory activity. Patent application WO 90/15602 describes pharmaceutical compositions useful in the treatment of inflammatory diseases, containing a N-[(9H-fluoren-9-yl-methoxy)carbonyl]-aminoacid as active ingredient, while patent application WO 91/18596 discloses pharmaceutical compositions containing fluorenyl compounds derivatized with a lipophilic amino acid or an alcohol, an amide or an ester derivative thereof. Further, patent application WO 93/11764 relates to fluorenyl-derivates of antinflammatory aminobenzoic acids.

It has been now surprisingly found that the antinflammatory activity of the fluorenylaminoacids dramatically increases as the compounds are derivatized with hydroxamic acids, thus also yielding an immunosuppressive activity.

Therefore the present invention refers to compounds of formula I



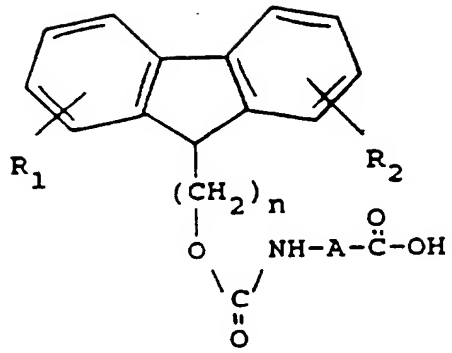
I

wherein n is an integer from 0 to 3; R_1 and R_2 are independently hydrogen, methyl, (C_{2-4})alkyl, halogen, hydroxy, hydroxymethyl, hydroxy(C_{2-4})alkyl, (C_{1-4})alkoxy;

A is methylene, linear or branched (C_{2-6})alkylidene, phenyl-methylidene, phenyl(C_{2-6})alkylidene, phenylene, naphthylene; and their diastereomers and enantiomers and the mixture thereof.

A (C_{1-4})alkyl group essentially identifies methyl, ethyl, propyl, i-propyl, butyl, 2-methyl-propyl, and a (C_{2-6})alkylidene is, e.g., ethylidene, propylidene, butylidene, isobutylidene, 2-methyl-butylidene, hexylidene, 2,3-dimethyl-butylidene and the like. Finally, an alkoxy group identifies methoxy, ethoxy, propoxy, isopropoxy, butoxy, 2-methylbutoxy and tert-butoxy.

The compounds of formula I may be prepared starting from a compound of formula II



II

wherein R_1, R_2, A and n are as described above, which is reacted with stoichiometric or slightly exceeding amounts of a carboxylic acid activator such as 1,1-carbonyldiimidazole, at a temperature varying from about -10°C to about 40°C, for about 2-48 hours, in the presence of etheric solvents such as tetrahydrofuran, 1,4-dioxane, diglyme, or in aprotic dipolar solvents such as dimethylformamide. The so activated compound of formula II is added with a stoichiometric or slightly exceeding amount of hydroxylamine hydrochloride, and the mixture is left to react at a temperature between about 0 and about 30°C, for about 1-20 hours, optionally in the presence of an equivalent of a tertiary organic base such as triethylamine or pyridine. The products of formula I thus obtained may be isolated and purified through techniques known by those skilled in the art.

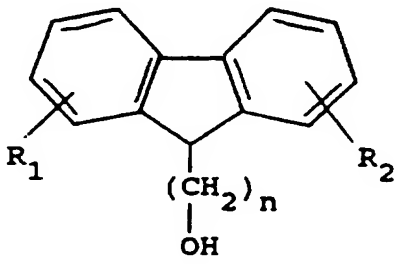
Where in the compound of formula II R_1 and/or R_2 are groups prone to react with the reactants used to prepare the compounds of formula I, said groups are suitably protected before such reaction and deprotected at the end of the same according to the teachings of the art (cfr., for example, T.W. Greene and P.G.M. Wuts - "Protective groups in organic synthesis", 2° ed., J.

Wiley & Son, 1991).

The compounds of formula II are known and/or commercially available products, the synthesis of which is described or deducible from the literature. See, for example, patents US-3.835.175 and US-3.906.031.

The preparation of the compounds of formula II implies obtaining an alcohol intermediate, the synthesis of which varies in view of the meaning of n.

When a compound of formula II wherein n=1 is desired, its synthesis starts from 9H-fluorene which, for example, is condensed with a suitable formic acid ester in the presence of a base such as sodium ethoxide, sodium hydride or sodium amide, and subsequently the aldehyde intermediate is reduced to yield the corresponding alcohol of formula (III)



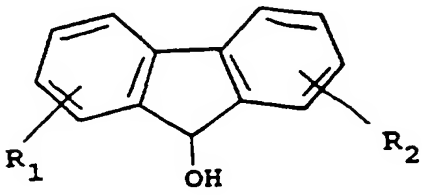
III

wherein R_1 and R_2 are as above, and n is 1.

Alternatively, 9H-fluorene may be directly condensed with formaldehyde in the presence of a strong base such as sodium hydride, sodium ethoxide or sodium amide, to yield the alcohol derivative III wherein n=1.

When a compound of formula II wherein n=0 is desired, 9-fluorenone is employed as reactant, and is reduced, for example, with a alkali metal hydride such

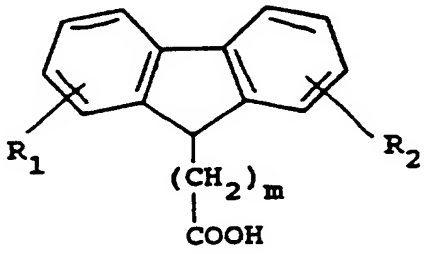
as sodium borohydride or lithium-aluminium hydride, to provide an alcohol derivative of formula IV



IV

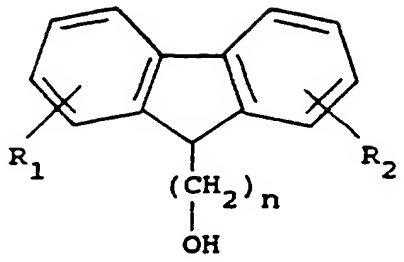
wherein R_1 and R_2 are as defined above.

Finally, if a compound of formula II wherein n is 2 or 3 is desired, the synthesis starts from the corresponding 9-fluoren-alkanoic acid of formula V



V

wherein R_1 and R_2 are as above and m is 1 or 2. Said acids are known as commercially available or described by the literature (cfr. Gualtieri F., J. Med. Chem., 28, No.11, 1621-1628, 1985). The acid of formula V is reduced with a suitable reducing agent such as lithium-aluminium hydride, to yield the alcohol derivative of formula III

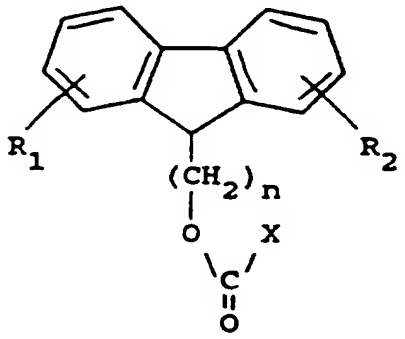


III

wherein R_1 and R_2 are as above, and n is 2 or 3.

If required, the insertion of the substituents R_1 and R_2 may be carried out using known procedures, for example, by direct alkylation or halogenation.

Using techniques generally known by the skilled artisan, the alcohol derivative of formula III or IV is converted in an intermediate of formula VI



VI

wherein R_1, R_2 and n are as defined above, and X is a leaving group which may be easily displaced by the nucleophilic nitrogen atom of an amino acid, for example an halogen atom, particularly chlorine or bromine, a lower alkoxy, mercapto, alkyl-mercapto group or succinylimidoyloxy. The latter is reacted with an amino-carboxy acid of formula VII

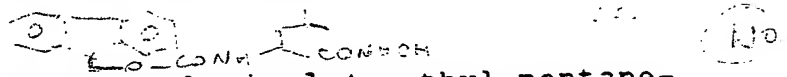
$$NH_2\text{-A-COOH} \qquad \text{VII}$$

wherein A is as defined above, to give the compound of

formula II. When the leaving group X is a halogen atom, particularly chlorine, the reaction is advantageously carried out in a polar organic solvent such as dioxane, tetrahydrofuran, dimethylformamide or pyridine, under alkaline, preferably mild, conditions, and at low temperature, for example from about 0°C to about 25°C, for a period of about 2-3 hours. In the case of other leaving groups X, a higher reaction temperature, for example of about 25-50°C, and longer reaction times, for example from about 8 to about 48 hours could be necessary. Generally, the compounds of formula II precipitate from the reaction mixture and may be purified, for example, by crystallization.

The ^{1}H-NMR spectra were made in dimethylsulphoxyde (DMSO) with a VARIAN GEMINI 200 spectrometer. The ^{13}C-NMR spectra were made using a VARIAN GEMINI 200 spectrometer (reference: 39.5 ppm peak of DMSO).

EXAMPLE 1



2-[(Fluoren-9-yl)methoxycarbonylamino]-4-methyl-pentane-hydroxamic acid

1.5 g (4.25 mmoles) of N-[(fluoren-9-yl)methoxycarbonyl]-(L)-leucine (Nova Biochem 04-12-1025, NPC 15199; WO 90/15602, Example 2) was dissolved in 17 ml of dry tetrahydrofuran (THF) and added, at 0°C, with 826 mg (5,1 mmoles) of 1,1'-carbonyldiimidazole (CDI). The mixture was left at room temperature per 10 hours, then added with 443 mg (6.37 mmoles) of hydroxylamine hydrochloride and left at room temperature for 16 hours. 20 ml of water were then added thereto, the liquor was saturated with sodium chloride, the phases separated and the organic one washed with brine (2x20 ml), then dried

over sodium sulfate and filtered. Evaporating the solvent under reduced pressure, a crude was obtained which was suspended under stirring in 20 ml of ethyl acetate for 4 hours, then filtered and dried to yield 900 mg of the title product.

m.p.. = 153°C (ethyl acetate).

Elemental analysis	C	H	N
calculated	68.46	6.57	7.60
found	68.15	6.51	7.72

^{1}H-NMR: s (1H) 10.16; s (1H) 8.85; d (2H) 7.91; d (2H) 7.75; d (1H) 7.57; m (4H) 7.47÷7.30; m (3H) 4.25; m (1H) 4.00; m (3H) 1.70÷1.31; d (3H) 0.90; d (3H) 0.86.

^{13}C-NMR: 169.14; 156.05; 144.21; 144.05; 140.97 (2C); 127.91 (2C); 127.33 (2C); 125.64 (2C); 120.37 (2C); 65.89; 51.00; 46.93; 41.13; 24.42; 23.10; 21.96.

EXAMPLE 2

[(Fluoren-9-yl)methoxycarbonylaminol-aceto-hydroxamic acid

Substantially following the steps of Example 1, starting from 2 g (6.7 mmoles) of N-[(fluoren-9-yl)-methoxy-carbonyl]-glycine (Nova Biochem 04-12-1001, NPC 14692) dissolved in 27 ml dry THF, 1.32 g (8.1 mmoles) of CDI and 698 mg (10 mmoles) of hydroxylamine hydrochloride, 800 mg of the title product were obtained.

m.p. 132°C dec (ethyl acetate)

Elemental analysis	C	H	N
calculated	65.38	5.16	8.97
found	66.73	5.06	8.80

^{1}H-NMR: s (1H) 10.49; s (1H) 8.80; d (2H) 7.90; d (2H) 7.73; t (1H) 7.58; m (4H) 7.47÷7.30; m (3H) 4.26; d (2H) 3.53.

^{13}C-NMR: 166.32; 156.68; 144.12 (2C); 140.97 (2C); 127.90 (2C); 127.56 (2C); 127.34 (2C); 125.54 (2C); 120.37 (2C); 65.99; 46.49; 41.60.

EXAMPLE 3

[(2,7-Dimethyl-fluoren-9-yl)methoxycarbonylamino]-4-methyl-pentane-hydroxamic acid

Starting from 1.25 g (3.28 moles) of N-[(2,7-dimethyl-fluoren-9-yl)methoxycarbonyl]-(L)leucine (WO 91/18596, Example 26; NPC 15669), dissolved in 20 ml of THF, 640 mg (3.9 mmoles) of CDI and 342 mg (4.9 mmoles) of hydroxylamine hydrochloride, and substantially following the procedure of Example 1, 980 mg of the title product were obtained.

m.p.. = 146-147°C (ethyl acetate).

Elemental analysis	C	H	N
calculated	69.68	7.12	7.07
found	68.69	6.97	6.92

^{1}H-NMR: s (1H) 10.63; s (1H) 8.81; s (1H) 7.73; s (1H) 7.69; d (1H) 7.56; d (2H) 7.52; d (2H) 7.21; m (3H) 4.31÷4.06; m (1H) 3.97; s (6H) 2.39; m (3H) 1.68÷1.29; d (3H) 0.90; d (3H) 0.87.

^{13}C-NMR: 169.12; 156.06; 144.35; 144.08; 138.45 (2C); 136.21 (2C); 128.52 (2C); 126.14 (2C); 119.72 (2C); 66.02; 51.01; 46.66; 41.20; 24.25; 23.09; 21.99; 21.49 (2C).

EXAMPLE 4

4-[2-(Fluoren-9-yl)ethoxycarbonylamino]-benzo-hydroxamic acid

A] 5 g (22.3 mmoles) of 9-fluoren-acetic acid (Aldrich) in 160 ml of THF, were added, at 0°C, with 1.7 g (44.6 mmoles) of lithium-aluminium hydride, then the reaction mixture was left at 25°C over night, treated with 0.47 ml of water, 1.27 ml of 20% sodium hydroxide, then again with 4.9 ml of water. The formed salts were filtered off, and the organic phase was dried under reduced pressure and gave 4.85 g of a crude which was purified by flash chromatography (eluent ethyl acetate/hexane 3:7) and gave 2.5 g of 2-(fluoren-9-yl)-ethanol.

B] 2.5 g (11.9 mmoles) of the compound under A], in 120 ml of acetonitrile, were sequentially added with 4.57 g (17.9 mmoles) of N,N'-disuccinimidyl carbonate and 0.9 ml (11.3 mmoles) of pyridine. The mixture was left at 25°C for 30 hours, then dried under reduced pressure at cold temperature. The crude was dissolved in 100 ml of ethyl acetate, washed with 1N hydrochloric acid (2x50 ml), and the organic phase was dried over sodium sulphate and dried under reduced pressure at cold temperature to yield 4.8 g of N-[2-(fluoren-9-yl)ethoxycarbonyloxy]-succinimide which was used as such in the next step.

C] A solution of 1.63 g (11.9 mmoles) of sodium carbonate in 24 ml of water was added with 1.63 g (11.9 mmoles) of 4-amino-benzoic acid and 4.8 g of the crude under B] dissolved in 24 ml of dioxane. After 3 days under stirring at room temperature, 100 ml of THF were added therein and, after saturation with sodium chloride, the phases were

separated and the organic one was dried after washing with brine (2x50 ml) and drying over sodium sulphate. The resulting solid was suspended in ethyl acetate, filtered and dried to yield 2.95 g of 4-[2-(fluoren-9-yl)ethyloxy-carbonyl]amino-benzoic acid.

D] 2.95 g (7.9 mmoles) of the compound under C] were dissolved in 32 ml of dry THF and treated substantially according to the procedure of Example 1, with 1.53 g (9.4 mmoles) of CDI and 825 mg (11.8 mmoles) of hydroxylamine hydrochloride. At the end of the reaction a deposit formed and was filtered, dried and suspended again in 1N hydrochloric acid under stirring for 10 hours. The suspension was washed with water and dried to yield 550 mg of the title product.

m.p. 200-203°C (water)

Elemental analysis	C	H	N
calculated	71.12	5.12	7.21
found	69.10	5.13	7.12

^{1}H-NMR: s (1H) 11.04; s (1H) 9.87; s (1H) 8.93; m (2H) 7.92; m (4H) 7.73÷7.64; m (6H) 7.53÷7.33; t (1H) 4.17; t (2H) 4.05; m (2H) 2.36.

^{13}C-NMR: 164.27; 153.56; 146.47 (2C); 142.03; 140.64 (2C); 128.00 (2C); 127.53 (2C); 127.45 (2C); 126.72; 124.79 (2C); 120.40 (2C); 117.68 (2C); 62.12; 44.07; 31.96.

EXAMPLE 5

2-[(Fluoren-9-yl)methoxycarbonylamino]-3-phenyl-propane-hydroxammic acid

3 g of N-[(fluoren-9-yl)methoxy-carbonyl]-(L)phe-

nylalanine (Nova Biochem 04-12-1030; WO 90/15602, Example 1) in 31 ml of dry THF were treated substantially following the procedure of Example 4,D] with 1.5 g (9.2 mmoles) of CDI and 800 mg (11.5 mmoles) of hydroxylamine hydrochloride to yield 1.5 g of the title product.

m.p. 185°C dec

Elemental analysis	C	H	N
calculated	71.63	5.51	6.96
found	71.44	5.59	6.94

^{1}H-NMR: s (1H) 10.73; s (1H) 8.88; d (2H) 7.91; d (1H) 7.77; m (2H) 7.68; m (9H) 7.47÷7.18; m (2H) 2.90.

^{13}C-NMR: 168.40; 155.95; 144.03 (2C); 140.92 (2C); 138.27; 129.49 (2C); 128.36 (2C); 127.91 (2C); 127.33 (2C); 126.58; 125.61 (2C); 120.36 (2C); 65.98; 54.29; 46.82; 37.96.

As already said above, the compounds of formula I showed to be endowed with immunosuppressive and antinflammatory activity.

Test 1

The immunosuppressive activity of the present compounds was evaluated by the test of the mixed lymphocitary response, as follows.

Spleens were taken from Balb/C and DBA/2 mice. The splenocytes was suspended in a culture medium (RPMI 1640, 50 µg/ml of gentamicin, 2 mM glutamine, non essential amino acids, sodium pyruvate, 10-5 β-mercaptoethanol, 5% bovine foetal serum, 10 mM Hepes) at a concentration of 5x105 cells/ml. 0.1 ml of splenocytes Balb/C was placed in 96-well plates with 0.1 ml of

splenocytes DBA/2 in a ratio of 1:1 stimulator/receptor. At time 0, some compounds of the invention and of the prior art were added at a concentration of from 3.12 to 100 µM, and the plates were placed in an incubator at 37°C under 5% of carbon dioxide. After 3 days the cells were labelled with tritiated thymidine (1 µCi/well) for 18 hours, then the radioactivity was evaluated by a "cell-harvester" and a β-counter.

The results are shown in the following Table as concentration inhibiting of 50% the immune response (IC_{50}).

TABLE

Example	IC_{50} (µM)	Prior art compound	IC_{50} (µM)
1	5.4	NPC 15199	53.5
2	8.6	NPC 14692	>100
3	2.1	NPC 15669	50.7
4	0.6	NPC 16570	9.6
5	13.3	Nova Biochem 04-12-1030	51.5

legenda:

NPC 15199 = N-(fluoren-9-yl)methoxycarbonyl-(L)-leucine (WO 90/15602, Example 2)

NPC 14692 = N-(fluoren-9-yl)-methoxy-carbonyl-glycine (WO 90/15602, Nova Biochem 04-12-1001)

NPC 15669 = N-(2,7-dimethyl-fluoren-9-yl)methoxycarbonyl(L)leucine (WO 91/18596, Example 26).

NPC 16570 = 4-[2-(fluoren-9-yl)ethyloxy-carbonyl]aminobenzoic acid (WO 93/11764, Example 7).

Nova Biochem 04-12-1030 = N-[(fluoren-9-yl)methoxy-carbonyl]-(L)phenylalanine (WO 90/15602, Example 1)

Test 2

The antinflammatory activity of the compounds of the invention was evaluated by the test of the polymorphonuclear leukocyte adhesion to endothelial cells, as described hereinbelow.

The polymorphonuclears (PMN) were obtained by separation from the blood of a healthy donor through sedimentation with Eufusin (Stholl Farmaceutici) followed by lysis of the erythrocytes with a solution of ammonium chloride. The PMN thus obtained were labelled with 51Cr (total incorporation = 12.303±377 cpm) and pre-treated with the compounds of the invention and of the prior art (each at a concentration of 100 µM, diluted in a medium containing 10% calf foetal serum and 0.1% DMSO) for 20 minutes at 37°C, then activated with 1000 U/ml of TNF for the following 10 minutes, finally were placed on the monolayer of endothelial human cells ("Human Umbelical Vein Endothelial Cell from 2nd to 4th passage) in 96-well plates, and incubated at 37°C with 5% CO2. After 15 minutes, the not adhered PMN were removed and a solution of 1N sodium hydroxide was added to each well to remove the adherent PMN. The radioactivity of the lysate was measured by a γ-counter ed expressed in cpm (count per minute).

The inhibition percentage of the cellular adhesion was calculated according to the following formula:

$$100 - \left[\frac{\text{cpm compound}}{\text{cpm control}} \times 100 \right]$$

wherein the control is the culture treated with TNF and medium containing 10% calf foetal serum and 0.1% DMSO only.

The compounds of the invention showed an inhibition of the cellular adhesion of about 70-90%, whereas the ones of the prior are of about 40-60%. For example, the compound of Example 3 provided an inhibition percentage of 77.4%, and the corresponding compound of the prior art, NPC 15669, of 58.2%.

Test 3

The toxicity of the compounds of the present invention was evaluated by an acute toxicity model in mouse after intraperitoneal administration. For example, The compound of Example 1 showed a LD50 >500 mg/kg whereas the corresponding compound of the prior art, NPC 12199, has a LD_{50} = 268 mg/kg.

In view of what above, the compounds of the invention showed to be useful in pathologies such as graft versus host disease, atopic and contact dermatitis, osteoarthritis, psoriasis, rheumatoid arthritis, glomerulonephritis, irritable bowel syndrome, lupus erythematosus, scleroderma, asthma, and, in general, all of those pathologies stimulating the lymphocitary response, or which are characterized by an inflammatory phenomenon.

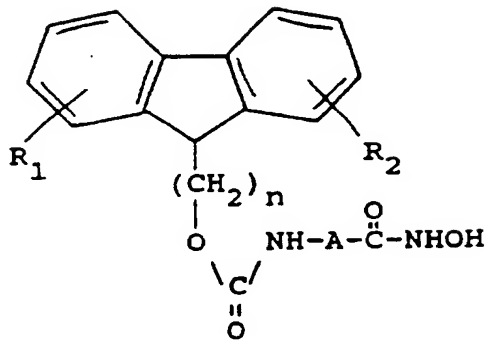
Object of the invention is also the use of the compounds of formula (I) as immunosuppressant and antinflammatory agents, and the industrial aspects connected to said use, comprising their incorporation into pharmaceutical compositions. Examples of pharmaceutical compositions are tablets, sugar- and film-coated tablets, sy-

rups, creams, ointments, suppositories, and phials, the latter being suitable both for the oral and the intramuscular or intravenous administration. They contain the active principle alone or in combination with common pharmaceutically acceptable carriers and excipients.

The dosages of active principle used to relieve and heal the inflammation may vary within wide limits according to the nature of the compound employed, of the pathology and of the condition of the patient (sex, age, general physical condition).

CLAIMS

1. Compounds of formula I



I

wherein n is an integer from 0 to 3; R_1 and R_2 are independently hydrogen, methyl, (C_{2-4})alkyl, halogen, hydroxy, hydroxymethyl, hydroxy(C_{2-4})alkyl, (C_{1-4})alkoxy; A represents methylene, linear or branched (C_{2-6})alkylidene, phenyl-methylidene, phenyl(C_{2-6})-alkylidene, phenylene, naphthylene; and their possible diastereomers and enantiomers and the mixtures thereof.

2. Use of the compounds of formula I for the preparation of a medicament useful in the treatment of inflammation, and when an immunosuppressant is requested.

3. Pharmaceutical compositions containing as active ingredient at least one of the compounds of formula I, together with pharmaceutically acceptable excipients.

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INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/EP 95/04388

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07C271/22 C07C271/28 A61K31/325

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07C A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,91 18596 (WEITZBERG MOSHE ;BURCH RONALD MARTIN (US)) 12 December 1991 cited in the application see claims	1-3
A	WO,A,92 02532 (GENTA INC) 20 February 1992 see claims; figures	1
A	EP,A,0 129 075 (HOFFMANN LA ROCHE) 27 December 1984 see claims	1

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
15 February 1996	23.02.1996

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Sánchez García, J.M.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/EP 95/04388

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9118596	12-12-91	US-A-	5079260	07-01-92
		AU-B-	7951591	31-12-91
		EP-A-	0531443	17-03-93
		JP-T-	5509299	22-12-93
		NZ-A-	238272	25-03-94
WO-A-9202532	20-02-92	AU-B-	664542	23-11-95
		AU-B-	9071091	02-03-92
		CA-A-	2089087	10-02-92
		EP-A-	0542928	26-05-93
		NZ-A-	239250	23-12-93
EP-A-0129075	27-12-84	JP-A-	59231061	25-12-84
		US-A-	4822890	18-04-89

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